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This is Exhibit SMT-5 referred to in the Statutory Declaration by Stephen Maxwell Taylor

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Before me:

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Local therapy with soluble complement receptor 1 (sCR1) suppresses inflammation in rat mono-articular arthritis

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SUMMARY

Complement activation has been implicated in the pathogenesis of human rheumatoid arthritis. We sought to determine whether inhibition of complement (C) using sCR1 could influence the development and progression of antigen arthritis in the rat, a recognized model of human chronic synovitis. The effect of C inhibition, systemically and locally, on three different stages of disease was examined: (i) prophylaxis, (ii) treatment of established inflammation, and (iii) prevention of antigen-induced flares of disease. Arthritis was assessed by knee swelling and by histological examination. Our results show that intra-articular injection of sCR1 prior to disease onset reduced joint swelling and development of arthritis, whereas systemic administration was ineffective. Treatment of established arthritis with intra-articular sCR1 3 days after disease onset caused a transient reduction in swelling, but treatment 7 days after disease onset had no effect on disease. An intra-articular dose of sCR1 given at the time of disease flares had a small, yet significant effect on knee swelling. We conclude that complement activation is important in the initiation and maintenance of inflammation in antigen arthritis. The potent effect of local C inhibition suggests that C biosynthesis and activation within the joint contributes to inflammation in this model of arthritis.

Keywords antigen arthritis rat complement sCR1

INTRODUCTION

Activation of complement (C) has been shown to contribute to tissue damage in a variety of inflammatory and infectious diseases [1]. Evidence of an involvement of C in rheumatoid arthritis (RA) has come from several sources; C activation products are present in blood and synovial fluid of patients with active RA [2–7], and products of C activation are deposited in the rheumatoid synovium. Human rheumatoid synovial cells attacked *in vitro* with C are induced to release a spectrum of inflammatory products including prostaglandins and cytokines [8,9], providing a mechanism for the perpetuation of joint inflammation. The development of a recombinant soluble form of the human membrane complement regulatory molecule, complement receptor one (sCR1:TP10) has been described [10]. This 247-kD protein is a potent inhibitor of the classical and alternative complement pathways. It binds to C3b and C4b, resulting in inactivation of the C3 and C5 convertase. It has proved an excellent inhibitor of the *in vitro* activation of serum complement in humans and a variety of other species. Since its first description, sCR1 has been shown to suppress tissue injury in a variety of animal models of human disease, including myocardial [11] and gut [12] ischaemia/reperfusion injury, graft rejection [13], adult respiratory distress syndrome [14] and experimental allergic encephalomyelitis [15]. This study describes the effect of sCR1 on the clinical course and pathogenesis of rat antigen arthritis, an animal model of human chronic synovitis. We show that intra-articular injection of sCR1 markedly reduced joint swelling and development of destructive arthritis, the latter judged by assessing the neutrophil infiltrate into the synovium and erosion of articular cartilage. Systemic sCR1 caused little inhibition of this arthritis, and the combination of therapies was not significantly better than intra-articular therapy alone. These results suggest that C is involved in the generation of pathology in this model and suggest that intra-articular synthesis of C is important in the initiation and perpetuation of the inflammatory process.

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MATERIALS AND METHODS

Animals

Male Lewis rats (100–150 g) were obtained from Bantin and Kingman (The Field Station, Hull, UK) and housed in cages of

four at Biomedical Services U.W.C.M. They were allowed free access to food and water and kept in light/dark cycles of 12 h.

Arthritis induction

Arthritis was induced following an established method [16]. Briefly, on two occasions a week apart, an emulsion containing equal volumes of methylated bovine serum albumin (mBSA; 0·5 mg/ml; Sigma Chemical Co., St Louis, MO) and Freund's complete adjuvant (FCA; 0·25 mg *Mycobacterium tuberculosis*; Sigma) was injected subcutaneously into the backs of the animals. Fourteen days after this second subcutaneous injection (day 0), an intra-articular injection of mBSA (0·1 mg in 100 μl of saline) was given into the right knee of each animal. The left knee served as a control and received an equal volume of saline.

Treatment regimens

Cobra venom factor (CVF) was purified from the venom of the Naja Naja cobra (Sigma) by the method of Vogel & Muller-Eberhard [17] and was a gift from Dr J. P. Camilleri (U.W.C.M.).

Recombinant, endotoxin-free sCR1 (TP10) at a concentration of 4·95 mg/ml in sterile PBS pH 7·0 was provided by T Cell Sciences Inc. (Needham, MA).

The effect of C inhibition on three different stages of disease was examined: (i) prophylaxis, (ii) treatment of established inflammation, and (iii) prevention of antigen-induced flares of disease.

Prophylaxis. Animals were randomized into groups of five. Disease was induced in all animals. Each group of five received one of the following treatment regimens: (i) i.p. CVF 100 U 2 days before onset of disease; (ii) i.p. CVF 2 days before onset of disease and intra-articular sCR1 with the disease-initiating antigen; (iii) i.v. sCR1 20 mg/kg daily for 7 days starting 2 days before disease onset; (iv) i.v. and intra-articular sCR1; (v) intra-articular sCR1(200 μg) as a single dose with the disease-initiating antigen; (vi) control saline-treated group.

Treatment. Arthritis was induced as described above and animals were randomized into groups of five, each group of animals to receive one of the following regimens: (i) 200 μg of sCR1 given intra-articularly 3 days after disease onset; (ii) 200 μg sCR1 given intra-articularly 7 days after disease onset; (iii) untreated controls.

Flare reaction. Arthritis was induced as described previously. Fourteen days after disease onset a flare was induced in 10 animals by a second intra-articular injection of mBSA (0·1 mg) into the inflamed right knee. Animals were divided into two groups matched for disease severity, one group receiving an intra-articular dose of sCR1 ($n = 5$) with the boosting antigen, control rats ($n = 5$) receiving mBSA alone.

Haemolytic assays

The animals receiving systemic therapy were bled daily and the haemolytic activity of each plasma sample was measured by minor modifications of published methods to confirm complement inhibition [18]. Briefly, plasma samples were diluted in veronal buffer and incubated with a standard concentration of sheep erythrocytes sensitized with ambozeptor (rabbit anti-sheep globin; Behring Hounslow, UK). Results were expressed as a percentage of normal rat plasma haemolytic activity in the same system

Disease assessment

Clinical. Disease was assessed clinically by measuring the knee diameters of both inflamed (right) and non-inflamed (left) knees, with a Mitutoyo digital calliper. The measurements were done in triplicate by an independent observer blind to the treatment regimen. The swelling attributed to the antigenic challenge was expressed as the difference in millimetres between the mean readings of the right (inflamed) and left (normal) knee diameters.

Histological. Rats from different treatment groups were killed 14 days after arthritis induction. The knee joints were removed intact and fixed in formalin-buffered saline for 1 week before histological processing. Joints were decalcified and embedded in paraffin wax, then sectioned in the sagittal plane at 5 μm and stained with haematoxylin–eosin. All sections were coded before assessment to eliminate observer bias and subsequently scored by an independent observer. Sections were graded subjectively on a scale of 0–4: 0 = normal; 1 = minimal synovial infiltration; 2 = moderate synovial infiltrate with inflammatory exudate in joint space; 3 = severe synovial infiltration and inflammatory exudate; 4 = severe synovial infiltration, inflammatory exudate and full thickness cartilage destruction with bone erosion.

Statistical analysis

The one way analysis of variance (ANOVA) was used to determine the significance of differences between the knee diameters in different treatment groups. Student's *t*-test was used to determine the significance of differences between individual groups. The Wilcoxon sum of ranks test was used to determine the significance of differences in the histological scores of the knees.

RESULTS

Decomplementation with sCR1 and CVF

CVF given as a single i.p. dose reduced the haemolytic activity in serum to zero. The animals remained completely decomplemented for 5 days. In sCR1-treated animals haemolytic assays were performed 24 h after each dose. Haemolytic activity of serum was reduced to 60% of the haemolytic activity of serum at day 0. However, this is an underestimate of the level of decomplementation attained during the 24 h post-therapy. Figure 1 shows a clearance study in naive animals. The haemolytic activity of rat serum after a single i.v. dose of sCR1 (20 mg/kg per animal) fell rapidly to 20% of that of normal rat serum within the first 15 min post-i.v. injection, rose to 40% by 2 h, was maintained at this level

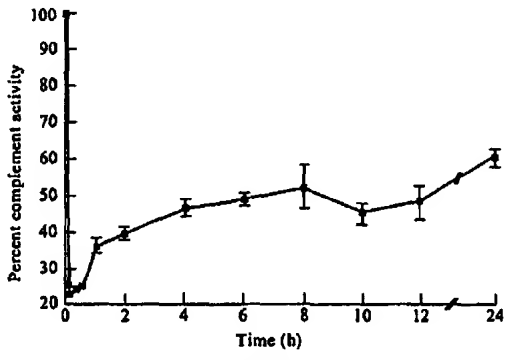

Fig. 1. Residual complement activity in rat serum after a single i.v. dose of sCR1 (20 mg/kg) over 24 h. Results are means of determinations in three naive rats, the vertical bar represents s.e.m. of the three measurements.

for a further 12 h and then slowly rose so that at 24 h the haemolytic activity was 60% that of normal rat serum. Animals receiving intra-articular sCR1 showed no evidence of systemic decomplementation (data not shown).

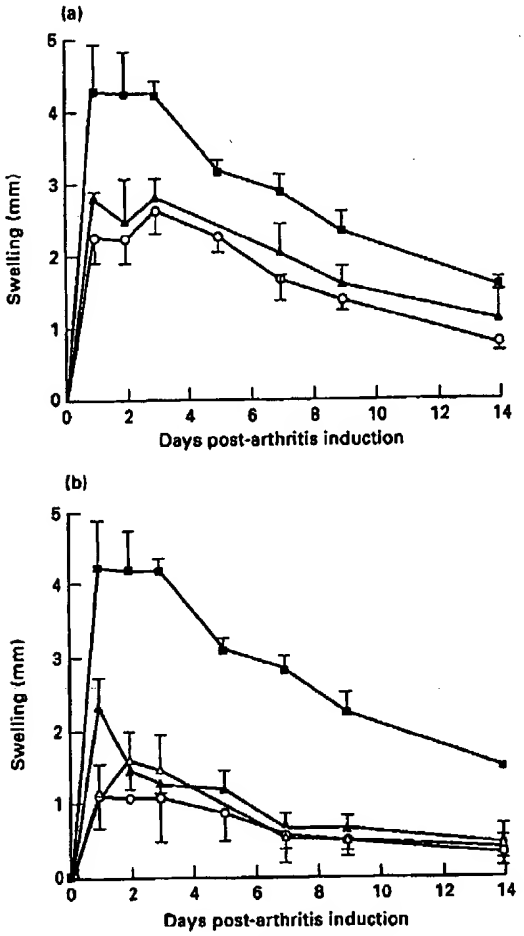

Fig. 2. (a) Effect of i.p. cobra venom factor (CVF) (▲), i.v. sCR1 (○), and saline (■) on the development of mono-articular arthritis in the Lewis rat. Significant difference ($P<0.05$) was seen in the knee swelling of the systemic CVF-treated group compared with controls on day 2; thereafter there was no significant difference. Similarly, significant difference was seen in the knee swelling in animals that received i.v. sCR1 compared with controls on day 1 ($P<0.05$), which became non-significant from day 7 until the end of the experiment. (b) Effect of intra-articular sCR1 (▲), i.v. and intra-articular sCR1 (○), i.p. CVF and intra-articular sCR1 (△) and saline (■) on the development of antigen arthritis in the Lewis rat. Results are means of five animals ± s.e.m. Significant differences were seen in the knee swelling of animals in all treated groups compared with controls throughout the experiment. There was no statistical difference between the knee swelling of animals in the groups that received systemic CVF or sCR1 in addition to intra-articular sCR1 and the group that received intra-articular therapy alone. Data are from a single study. The study was repeated three times with comparable results.

Effects of therapy on clinical disease

Prophylaxis. The effects on antigen arthritis of inhibition of C with either CVF or i.v. sCR1 are shown in Fig. 2a. CVF had a small but significant effect on the swelling in the first 2 days after antigenic challenge ($P<0.05$). Swelling was decreased compared with C-sufficient controls (2·75 mm compared with 4·2 mm). However, this effect was not sustained beyond 2 days, and the difference in swelling became non-significant for the remainder of the experiment. Intravenous sCR1 alone showed similar results, having a significant anti-inflammatory effect for 3 days.

The effects on antigen arthritis of intra-articular therapy with sCR1 are shown in Fig. 2b. A significant reduction in joint swelling in both groups that received intra-articular sCR1 was apparent 1 day after intra-articular challenge ($P<0.02$), which persisted for the duration of the experiment. The addition of systemic decomplementation using either CVF or sCR1 did not further enhance the anti-inflammatory effect of intra-articular therapy alone.

Treatment. Treatment of established arthritis with a single intra-articular dose of sCR1 3 days after disease onset caused a small, transient reduction in swelling which was significant at 24 h (Fig. 3). Treatment 7 days after disease onset, however, had no effect on the clinical disease (data not shown).

Treatment of a 'flare'. A repeat intra-articular challenge given 14 days after initiation of disease caused a flare in disease activity and increase in joint swelling (Fig. 4a). The flare was significantly dampened ($P<0.02$) in the group which received intra-articular sCR1 at the same time as the flare-inducing antigen. This difference persisted for 3 days after therapy. Identical results were seen when the flare was induced on established chronic disease, 40 days after disease initiation (Fig. 4b).

Histology

Table 1 summarizes the results of histological analyses of the fixed joint sections, and representative micrographs of the histological sections are shown in Fig. 5. The results mirror the change in knee

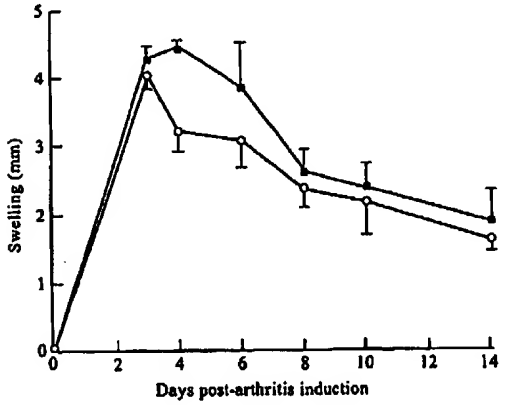

Fig. 3. Effect of treatment of established antigen arthritis with a single intra-articular dose of sCR1 given 3 days after development of disease. Results are means of five animals ± s.e.m. Significant differences in knee swelling between animals in the intra-articular sCR1-treated (○) and untreated control (■) groups was seen on day 4 (1 day after treatment) ($P<0.02$), which became non-significant thereafter. Data are from a single study. The study was repeated twice with comparable results.

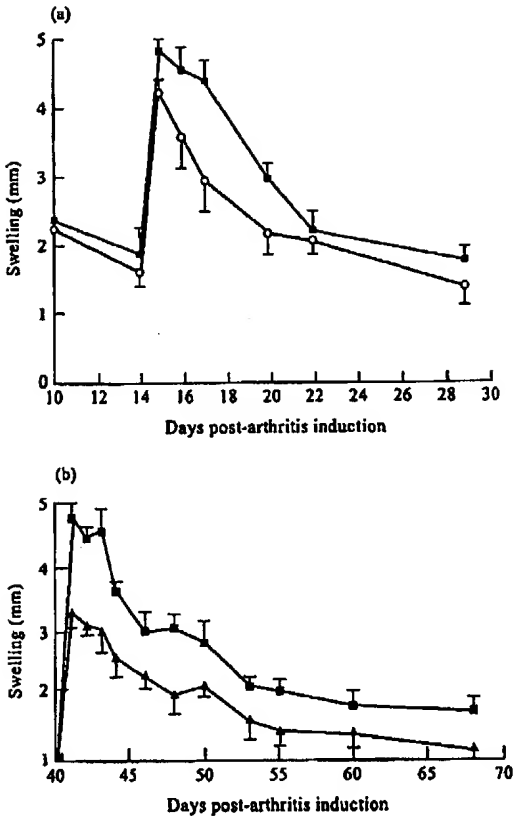

Fig. 4. Effect of a single dose of sCR1 on the development of a disease flare. (a) Flare was initiated on day 14 of already established arthritis by administration of a repeated dose of methylated bovine serum albumin (mBSA) to the right knee. Animals treated with a dose of intra-articular sCR1 at the time of flare induction (○) had significantly less knee swelling ($P<0.02$) from days 16 to 20 compared with animals that received mBSA alone (■). (b) Flare was initiated on day 40 of already established arthritis by administration of a repeated dose of mBSA to the right knee. Animals treated with a dose of intra-articular sCR1 at the time of flare induction (▲) had significantly less knee swelling ($P<0.05$) on days 42–44 compared with animals that received mBSA alone (■). Data are from a single study. The study was repeated twice with comparable results.

swelling. Comparison of the prophylactic effects of the different treatment regimens 14 days after arthritis onset revealed no statistically significant difference in the histological score between controls and those which received systemic therapy with either CVF or sCR1. However, intra-articular delivery of sCR1 improved the histological picture significantly ($P<0.0002$). The combination of both routes of therapy did not further enhance the effect of intra-articular therapy alone.

Table 1. The effect of complement inhibition upon the histological progression of antigen-induced arthritis in rats

Treatment protocol	Individual histological score
Untreated control	4, 3, 4, 4, 4, 4, 4, 3, 4
i.v. sCR1 (on day −2)	2, 2, 3, 2, 4
i.a. sCR1 (on day 0)*	0, 0, 0, 1, 0, 2, 3, 1, 0
i.v. sCR1 (on day −2) and i.a. sCR1 (on day 0)†	1, 0, 0, 1
i.p. CVF (on day −2)	4, 2, 3, 3, 2, 3, 1
i.p. CVF (on day −2) and i.a. sCR1 (on day 0)‡	1, 0, 0, 0, 1

Data are from a single study. The study was repeated three times with comparable results.

Individual histological scores, as assessed by a blinded observer, for the different treatment groups, are reported. When the scores from treated rats were compared with untreated controls by the Wilcoxon sum of ranks test:

*$P<0.002$ for intra-articular (i.a.) sCR1 administered on day 0.

†$P=0.05$ for combination therapy with sCR1 administered by i.v. injection on day −2 and i.a. sCR1 administered on day 0.

‡$P<0.002$ for combination therapy with cobra venom factor (CVF) administered by i.p. injection on day −2 and i.a. sCR1 administered on day 0.

DISCUSSION

RA is a chronic disease of unknown aetiology affecting principally synovial joints. The stimulus which maintains joint inflammation is unclear, but recent observation that the infusion of anti-tumour necrosis factor-alpha (TNF-α) MoAb suppresses joint inflammation in patients with RA highlights the importance of this macrophage-derived cytokine [19]. C-binding immune complexes or other C-derived inflammatory mediators are present within the joint and may also be important in initiating and perpetuating the inflammatory response contributing to joint damage [20–23].

The relevance of animal models to human disease has always been questioned. No animal model is strictly analogous to human RA, yet historically they have been used as proving grounds for potential therapeutic agents. Activation of the C system has been described in three recognized models of RA, namely, rat collagen-induced arthritis [24], streptococcal cell wall arthritis in rats [25], and, recently, cationic immune complex arthritis in mice [26]. It has been reported that the development of joint inflammation in each of these animal models is modified by complement depletion with CVF [24–26]. It is not, however, feasible to exploit this inhibitory effect and use this agent as a therapy in humans, as CVF is highly immunogenic and promotes the generation of proinflammatory mediators (C3a, C5a and the membrane attack complex) by activating the C pathway to completion. The development of safe, specific and potent C inhibitors such as sCR1 generated new interest in the potential manipulation of the C system in a range of inflammatory diseases.

We wished to test the effects of sCR1 in a rat model of arthritis. We chose antigen arthritis, a model in which the role of C has not previously been examined. Dumonde & Glynn [27] initially described antigen arthritis as a chronic inflammatory response in rabbits immunized with homologous and heterologous fibrin. Antigen arthritis has since been induced in rodents by immunization with a variety of antigens, such as ovalbumin [22], BSA [28] or mBSA [16], followed by the intra-articular injection of the same antigen. Antigen arthritis is a modified Arthus reaction, in which immune complexes are deposited in articular collagenous tissues, where they may activate C and other effector systems [20]. The model possesses several key similarities with human RA, notably

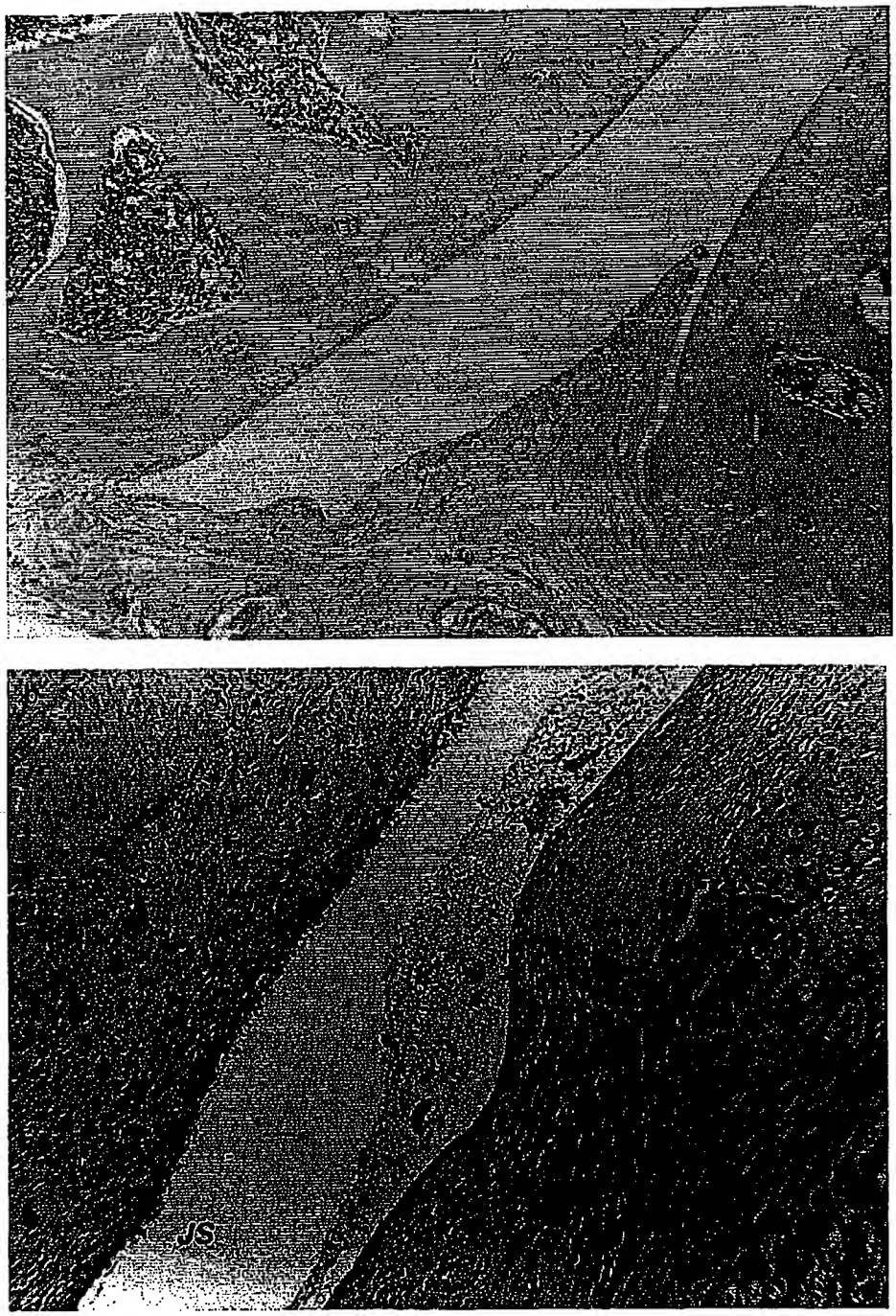

Fig. 5. (See next page.) Representative haematoxylin–eosin-stained sections of knee joints isolated from rats with antigen arthritis. (a) Intra-articular sCR1-treated rat showing no synovial infiltrate, histological score = 0. (b) Inflammatory exudate (IE) in the joint space (JS), histological score = 2.

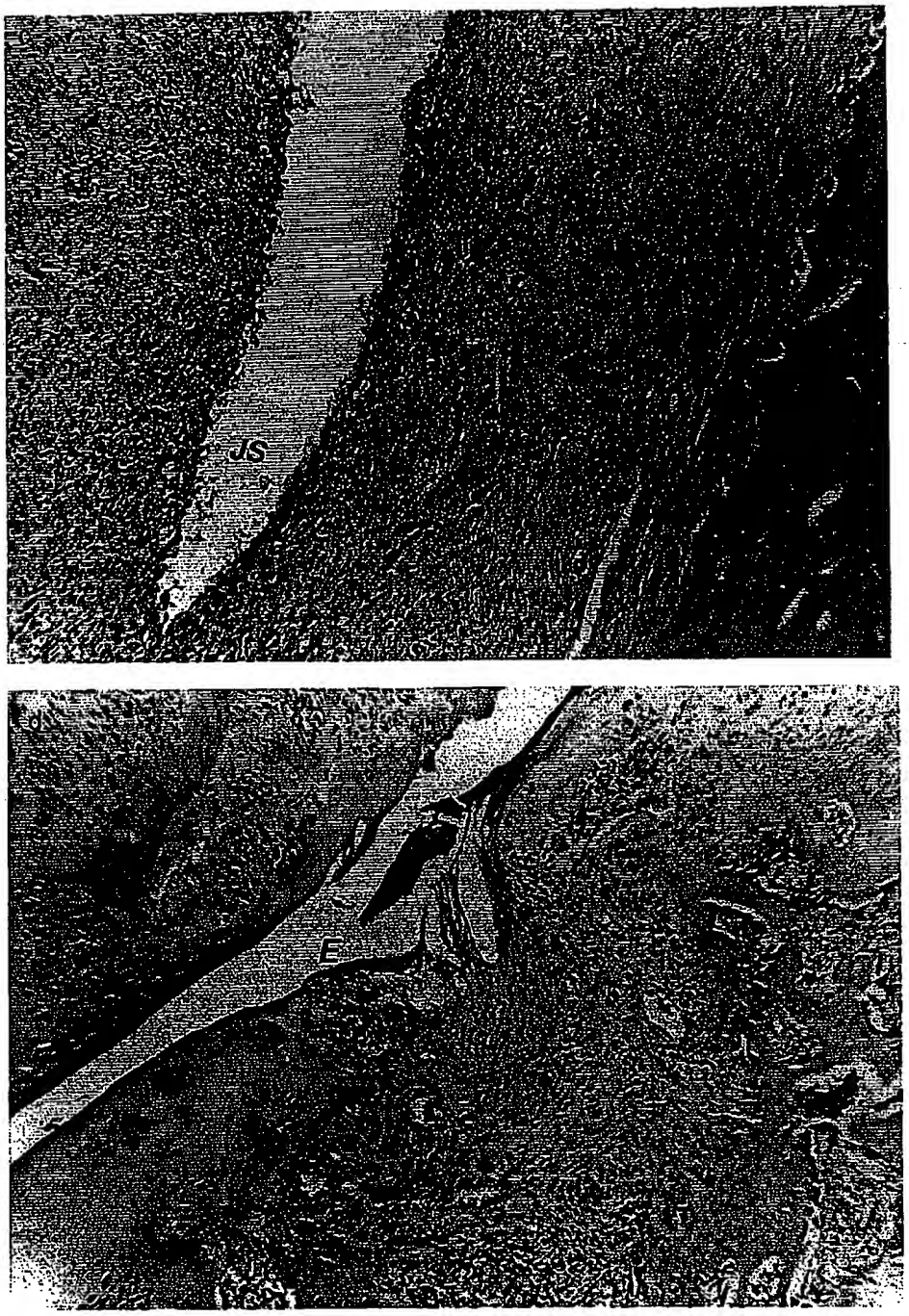

Fig. 5 (c) Severe synovial inflammatory infiltrate (SI) with no bone erosions, histological score = 3. (d) Untreated control rat showing synovial infiltrate and full thickness cartilage destruction and bone erosion (E); histological score = 4. (Mag. × 10.)

the deposition and persistence of immune complexes within the joint; the joint pathology which includes hyperplasia of synovium forming pannus and cartilage erosions, synovial biochemistry and response to anti-inflammatory drugs are also similar to those seen in human RA. We thus reasoned that this model was the best available for testing this potential therapy.

It was impossible to obtain complete inhibition of C by daily i.v. administration of sCR1, using doses and therapy schedules which have been reported by others to give complete inhibition of C in rats [15]. The reasons for this discrepancy are uncertain, but using standard assay systems we consistently found that the effects of sCR1 were short-lived (Fig. 1). Nevertheless, despite the incomplete inhibition of C we did observe a small, transient, but significant reduction in joint swelling (Fig. 2a). A recently published paper examining the effects of sCR1 on vascular injury and inflammation during renal allograft rejection in the rat showed identical kinetics of C inhibition, and despite the inability to cause sustained complete C inhibition a beneficial therapeutic effect was seen [38].

In order to eliminate completely the effects of systemic C, we used CVF, a single i.p. dose of which caused complete C inhibition for >3 days. The effects on joint inflammation were no greater than with the partial C inhibition caused by sCR1. Neutrophil accumulation within the joints was similar to that in untreated animals with both methods of inhibiting systemic C. This suggested either that C played a minor role in this model, or that C biosynthesis in the joint was involved. sCR1 is a large molecule with low tissue penetrance; it is thus unlikely to enter the joint in significant amounts. Therefore in order to explore the role of intra-articular C synthesis we examined the effects of intra-articular administration of sCR1 on the development of disease. Intra-articular therapy reduced joint swelling to a far greater degree than systemic decomplementation alone (Fig. 2b), whereas the combination of both i.v. and intra-articular routes did not have an additive effect. Importantly, intra-articular treatment with sCR1 also markedly inhibited inflammation and destructive changes in the joint (Table 1 and Fig. 5). The effects of intra-articular therapy suggest that the synovial space has the potential to behave as an isolated environment capable of producing C components, and that local C biosynthesis and activation is a driving force behind the inflammatory response. Such a scenario has been demonstrated in other tissue sites, e.g. kidney [29], brain [30], and suggested to occur in the joint [31]. Indeed, it has been proposed that local C synthesis may be more relevant to disease in numerous situations [32].

In normal and arthritic synovium there is a variety of cell types which are capable of producing C components. Infiltrating polymorphonuclear cells are capable of producing C3, C6, C7 [33], activated macrophages C1, C4, C2, C3, C5 [34], and resident synovial fibroblasts C3 and several other components [35–37]. As these cells are all present in the inflammatory pannus and at the sites of bony erosions, the local synthesis of C could contribute significantly to inflammation. In this context it is worth noting that a study of C3 metabolism in a patient with RA showed that approximately half the C3 present in the joint had been synthesized locally [38]. It has recently been shown that inhibition of the membrane regulator, CD59, in the normal rat knee joint triggers an acute transient inflammatory arthritis [31]. The membrane attack complex is deposited on the synovial cells even after systemic decomplementation, suggesting that the synovium is the source of C in this model, thus providing further evidence for the importance of local C biosynthesis in initiating joint inflammation.

Having demonstrated that C has a role to play in the induction of disease in this model, we proceeded to examine its role in established disease, as this more closely mimics the potential therapeutic option in human arthritis. Different results were seen depending on the timing of treatment, treating 3 days after onset of disease causing a significant, albeit small and transient, reduction in inflammation, whereas treating 7 days after disease onset did not influence the established inflammation. This suggests that different components of the immune system have different relative contributions to the persisting inflammation at different stages of disease. This supports the findings of Griffiths [16] in this disease model, who reported that different cellular populations predominate at different stages of disease, neutrophils predominating during the early stages, whereas macrophages and T cells were more abundant during the chronic phase.

Rheumatoid disease is a chronic disease characterized by recurrent exacerbations of joint inflammation. Unlike other models of arthritis, e.g. collagen-induced arthritis [24], the antigen-induced arthritis model offers the possibility to induce similar flares of disease by repeated application of the intra-articular antigen. Flare was induced 14 and 40 days after initial disease onset. The pathology at the time of flare induction is characterized by macrophages and T cells populating the joint in increasing numbers in contrast to the abundance of neutrophils in the acute disease [16]. Treating with intra-articular sCR1 had a small but significant beneficial effect on the swelling which persisted for 3 days. It did not, however, influence the ultimate course of the disease. The data suggest that during a flare of disease or repeat intra-articular challenge there is increased generation of immune complexes which trigger C activation, with the consequent recruitment of neutrophils and production of inflammatory mediators. Inhibition of C thus dampens the flare but has no long-term effect on the underlying chronic disease.

In conclusion, local inhibition of C with a single dose of intra-articular sCR1 had a beneficial effect on the course of antigen arthritis when given at the time of disease induction or during the induction of a flare of established disease. The effects observed on established disease and disease flares were modest and transient. However, sCR1 is a human C inhibitor, the efficacy of which is much reduced in rats [10]. It is possible that its more powerful inhibitory effect on human C and longer half-life in man will make it a much better inhibitor of human disease. The intra-articular route of administration is an attractive prospect, as the requirement of C for host defence against infection may preclude prolonged systemic inhibition of C as a therapeutic strategy. Local inhibition caused no reduction in systemic C activity, and offered additional therapeutic advantages as the doses required for local treatment were much lower than those necessary for systemic treatment. We suggest that local therapy with sCR1 offers an exciting prospect for treatment of human rheumatic disease.

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